A novel polysaccharide from Se-enriched *Ganoderma lucidum* induces apoptosis of human breast cancer cells

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Abstract. The novel polysaccharide SeGLP-2B-1 isolated from Se-enriched *Ganoderma lucidum*, showed anti-proliferative activity towards several cancer cell lines *in vitro*. To investigate the antitumor mechanisms, the apoptotic effects of SeGLP-2B-1 in human breast cancer cells were studied, and the mechanism of this action was further elucidated. Cell apoptosis was detected by Annexin V/PI staining. Caspase activity was assayed using a caspase apoptosis detection kit. Western blot analysis was used to evaluate the levels of pro-caspase-3, -8, -9, PARP and cytochrome *c* expression. The results showed that SeGLP-2B-1 inhibited the growth of MCF-7 cells in a time- and dose-dependent manner. Typical characteristics of apoptosis were observed, including morphological changes, sub-G1 cells and DNA ladder formation. Further analysis showed that SeGLP-2B-1 treatment disrupted the mitochondrial membrane potential followed by an increase in the cytochrome *c* cytosolic levels. Sequentially, SeGLP-2B-1 increased the activities of caspase-9, -3 and poly (ADP-ribose) polymerase in a time-dependent manner, however, no obvious activation of caspase-8 was observed. Caspase-9 and caspase-3 inhibitor prevented SeGLP-2B-1-induced apoptosis, and the activities of caspases-3, -9 were significantly up-regulated by SeGLP-2B-1. Our studies suggest that SeGLP-2B-1 induces apoptosis via a mitochondria-mediated pathway.

Introduction

Epidemiological studies suggest that dietary and chemo-prevention can provide an important and potentially feasible pathway for reducing cancer occurrence. Selenium (Se) is an essential trace element for a number of metabolically important enzymes (1). Accumulating evidence indicates that a signi-

ficant inverse relationship between intake of Se and cancer incidence and Se has received considerable attention for its potential role as a chemopreventive agent (2,3). Selenite induce cell necrosis accompanied by damage to cellular DNA and loss of cell-membrane integrity (4,5). Organic seleno-compounds induced apoptosis (programmed cell death) without producing changes in membrane integrity or cellular DNA as measured by single-strand breaks in DNA (6-8). Both organic and inorganic compounds of Se have been demonstrated to manifest cancer preventive potential, but several organic seleno-compounds have shown higher anti-cancer activities, lower toxicity and fewer side effects than selenite, therefore, it is crucial to develop suitable organic Se sources for cancer chemoprevention.

Se-containing polysaccharide, a nutritionally available organic seleno-compound, has been shown to promote cancer cell differentiation, suppress cell division and induce programmed cell death by regulating cancer gene expression (9,10). Although several mechanisms have been proposed to account for anticancer effects of Se-containing poly-saccharide, selective induction of tumor cell apoptosis may be of particular significance as chemopreventive agents (11-14).

SeGLP-2B-1, a Se-containing polysaccharide, was purified and characterized from the Se-enriched *Ganoderma lucidum* for the first time in our laboratory. Previous studies demonstrated that SeGLP-2B-1 exhibited strong antiproliferative activity against six human cancer cell lines in a Se-dependent manner (15). Treatment of cancer cells with SeGLP-2B-1 produced the typical characteristics of apoptosis, including cell shrinkage, compaction of cytoplasmic organelles, karyopyknosis, condensation and margination of nuclear chromatin, and cytoplasmic vacuolization. In our current study, we investigated the effects of SeGLP-2B-1 on cell growth inhibition and apoptosis in the breast cancer cell line MCF-7. We show that SeGLP-2B-1 indeed decreases cell viability and induces apoptosis. Our results also demonstrate that SeGLP-2B-1 induces MCF-7 cell apoptosis via a mito-chondrial pathway.

Materials and methods

Preparation of SeGLP-2B-1. SeGLP-2B-1 was prepared as described (15). SeGLP-2B-1 was isolated and identified from the mycelia of Se-enriched *G. lucidum* using a DEAE-52 column, a TSK-G5000PW HPLC column and a SHIM-

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PACK HPLC column. The polysaccharide content was determined with the phenol-sulfate method using glucose as the standard (16). The molecular weight of SeGLP-2B-1 was determined by HPSEC. The Se content of SeGLP-2B-1 was evaluated using inductively coupled plasma-atomic emission spectroscopy (Plasma-Spec-II, Leeman Labs) as reported (17). Standard bovine liver powder was the normal control.

Cell viability assay. Cell viability was assessed using the MTT assay (18). Briefly, MCF-7 cells were incubated in RPMI-1640 with 10% (v/v) heat-inactivated fetal calf serum in 96-well plates. After a 24-h incubation, SeGLP-2B-1 was added to each well to a final concentration ranging from 0.045 to 0.36 μM for 24, 48 or 72 h. At the end of the treatment, 20 μl of MTT solution was added to each well and incubated for an additional 4 h. After washing and centrifugation, 150 μl of DMSO was added to dissolve the MTT formazan crystals. The spectrophotometric absorbance at 592 nm was determined using a scanning multiwell spectrophotometer. In each experiment, the MTT assay was performed in eight replicates. Each experiment was repeated three times.

Annexin V/PI staining. Cells treated with 0.18 μM SeGLP-2B-1 were harvested and labeled with Annexin V/PI conjugated to fluorescein (Roche Diagnostics, Indianapolis, IN). The labeled cells were analyzed by flow cytometry on an EPICS®XL flow cytometer (Beckman Coulter, Fullerton, CA).

Cell cycle analysis. Cells incubated with 0.18 μM SeGLP-2B-1 were collected, washed, suspended in ice-cold PBS, gently fixed in 70% ice-cold ethanol at -20˚C for 24 h and stained with PI in a dark room at room temperature for 30 min. Cell cycle analysis was performed using an EPICS®XL flow cytometer, and the results were analyzed by the system-III software (Beckman Coulter). The proportion of sub-G1 hypodiploid cells was quantitated and presented as a percentage of apoptotic cells. The results are presented as the mean ± SD from three independent experiments.

Mitochondrial membrane potential (ΔΨm) assay. Cells treated with 0.18 μM SeGLP-2B-1 were stained with rhodamine123 and analyzed using a FACSCalibur flow cytometer with an excitation wavelength of 488 nm and an emission wavelength at 525 nm for green fluorescence. The mean fluorescence intensity (MFI) from rhodamine123 was used to indicate the ΔΨm of cells.

Western blot analysis. Cells treated with 0.18 μM SeGLP-2B-1 were washed and then lysed in 50 mM Tris-HCl, pH 8.0, containing 1 mM PMSF, 1 mM EDTA, 150 mM NaCl and 1% SDS. Cell lysates were obtained by centrifugation at 10000 x g for 40 min at 4˚C, and the protein content of the supernatant was determined using the Bradford method (19). Proteins were subjected to electrophoresis on 13.3% SDS-polyacrylamide gels and were subsequently transferred to a polyvinyl difluoride membrane. Membrane was blocked with non-fat milk in PBS containing 0.1% Tween-20, and then treated with primary antibodies overnight at 4˚C and horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse
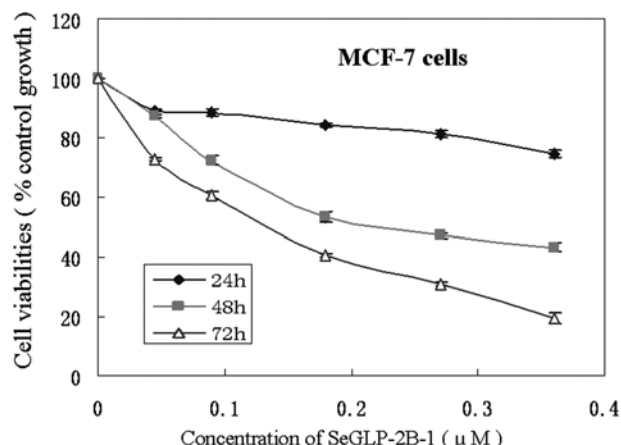

Figure 1. Effect of SeGLP-2B-1 on MCF-7 cell viability. MCF-7 cells were treated with SeGLP-2B-1 at concentrations from 0 to 0.36 μM for 24, 48 or 72 h. Cell viability assays were performed as described in Materials and methods. Results shown represent the mean ± SD (n=3).

IgG (1:5000) for 1 h at 30˚C. Immunoreactive bands were visualized using the ECL Western Blotting Detection system kit (Amersham Biosciences, Buckinghamshire, UK). ß-actin was detected as a control. The intensities of the protein bands were quantitated with densitometry using Scion Image software.

Caspase activity and effect of caspase inhibitors. Cells were treated with 0.18 μM SeGLP-2B-1 after preincubation with caspase inhibitor (caspase-3 inhibitor, z-DEVD-FMK; caspase-8 inhibitor, z-IETD-FMK; caspase-9 inhibitor, z-LEHD-FMK) for 1 h. Cell viability was evaluated using the MTT method. Caspase-3, -8 and -9 activities were measured using a Caspase Apoptosis Detection kit (Biovision Inc. Mountain View, CA).

Statistical analysis. The data were analyzed using one-way analysis of variance and Duncan's test. Triplicate samples were used to generate each data point. Statistical significance was set at p≤0.05.

Results

SeGLP-2B-1 reduces the viability of MCF-7 cells. The effect of SeGLP-2B-1 on MCF-7 cell viability was examined after cells were treated with increasing concentrations of SeGLP-2B-1 for 24-72 h. At 24 h, the cell viability of MCF-7 cells treated with SeGLP-2B-1 at various concentrations was not significantly decreased (Fig. 1). After 48 h, MCF-7 cell viability was significantly reduced in a dose-dependent manner; at 0.18 μM SeGLP-2B-1, the number of survival cells decreased by >50%.

SeGLP-2B-1 induces apoptosis in MCF-7 cells. To investigate whether the observed SeGLP-2B-1-induced decrease in MCF-7 cell viability was due to apoptosis, firstly, we assessed the formation of sub-G1 apoptotic bodies. The results showed that the proportion of sub-G1-phase cells increased over time (Fig. 2). Before treatment with 0.18 μM SeGLP-2B-1 for 24 h,
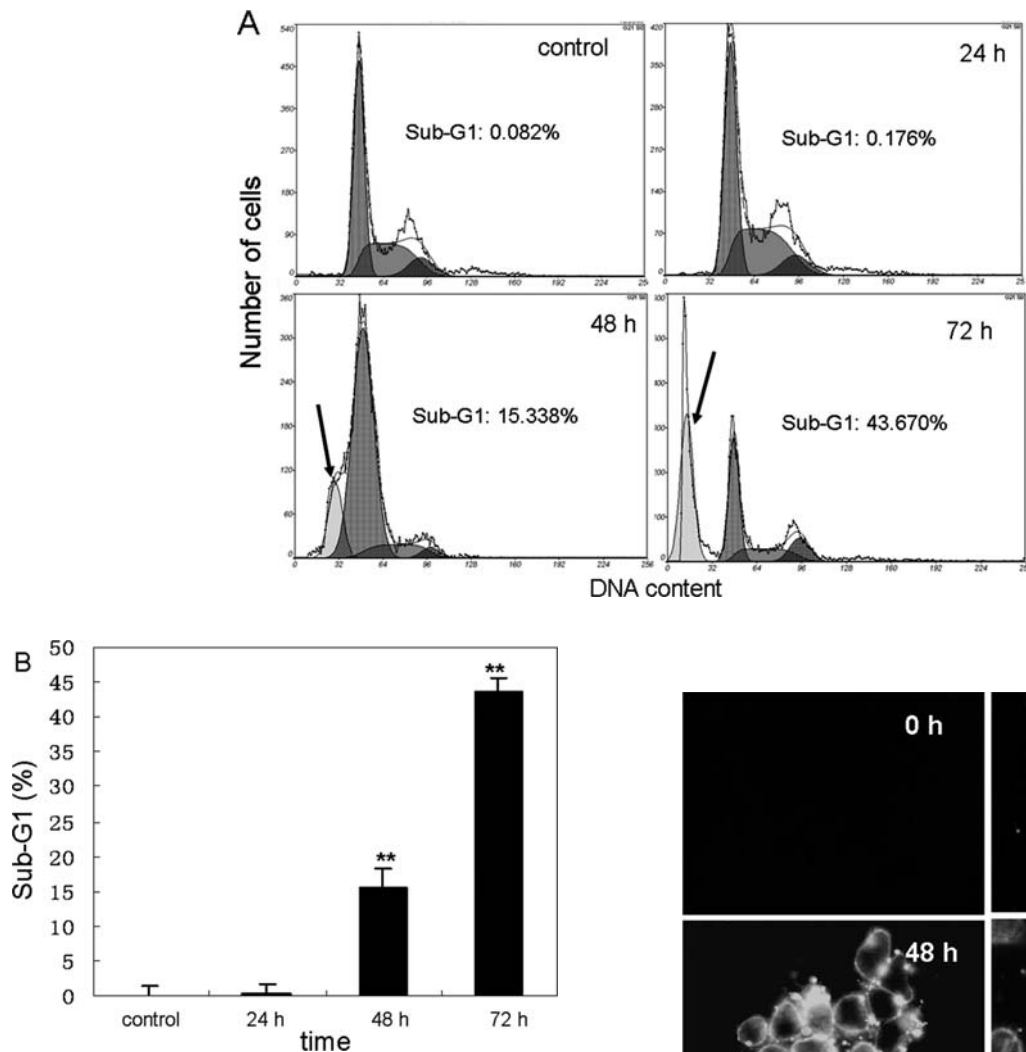
Figure 2. The formation of sub-G1 apoptotic bodies in MCF-7 cells. (A) MCF-7 cells treated with SeGLP-2B-1 were collected, fixed and stained with PI. The cell cycle phase distribution was analyzed using flow cytometry. The arrow indicates the sub-G0/G1 phase (apoptotic cell peak). (B) Quantitative analysis of the proportion of cells in the sub-G1 phase. Each value represents the mean of three independent experiments. **p≤0.01 compared with control group.
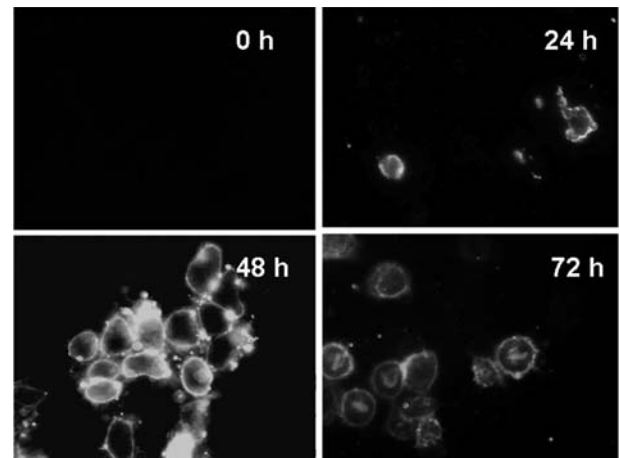

Figure 3. SeGLP-2B-1-induced apoptosis of MCF-7 cells. AV$^+$/PI$^-$ cells represent early apoptotic cells, and AV$^+$/PI$^+$ represent secondary necrotic cells.

no obvious sub-G1 peak (% apoptotic cells) was observed, but after incubation with SeGLP-2B-1 the proportion of apoptotic cells increased significantly (p≤0.05) from 0.082% (control) to 15.338% (48 h) and 43.670% (72 h). Analysis using Annexin V/PI staining further confirmed that SeGLP-2B-1 induced apoptosis. As shown in Fig. 3, few apoptotic cells were observed before treatment with 0.18 μM SeGLP-2B-1 for 24 h, but early apoptotic cells and secondary necrotic cells were present at 48 and 72 h, respectively.

Mitochondria dysfunction induced by SeGLP-2B-1. A variety of key events in apoptosis involve mitochondria, including loss of ΔΨm and the release of caspase activators such as cytochrome *c*. The disruption of mitochondrial integrity is one of the early events leading to apoptosis. To examine if mitochondria play a role in the observed SeGLP-2B-1-induced apoptosis, we first measured ΔΨm in MCF-7 cells treated with SeGLP-2B-1 using flow cytometry after rhodamine123

staining. As shown in Table I, the change of ΔΨm was observed in treated MCF-7 cells. Slight decrease of mean fluorescence intensity was detected in cells treated with 0.18 μM SeGLP-2B-1 at 24 h. Compared to control, the decrease in the mean fluorescence density was about 37.9-54.3% at 48 and 72 h, respectively. The results showed that SeGLP-2B-1 could induce the dissipation of mitochondrial ΔΨm in a time-dependent manner.

The release of cytochrome c. A reduction in the mitochondrial membrane potential is usually accompanied by release of cytochrome *c* into the cytosol. The release of cytochrome *c* from mitochondria is a particularly important event in the induction of apoptosis. Therefore, we examined the amount of cytochrome *c* in the cytoplasm using Western blotting. Cyto-chrome *c* was released from mitochondria, which resulted in a significant (p≤0.05) increase in cytosolic cytochrome *c*. No significant increase was seen within 24 h.

Table I. The disruption of $\Delta\Psi m$ in MCF-7 cells treated with SeGLP-2B-1 for 0-72 h.

	Mean fluorescence intensity (MFI)	Lost $\Delta\Psi m$ (% control)
Control	225.2±63.4	
24 h	208.8±13.5	7.2±3.2
48 h	119.7±56.2[a]	46.8±16.8[a]
72 h	103.6±37.5[a]	54.3±9.2[a]

Reported values are presented as the mean ± SD (n=3). [a]p<0.05 is significantly different from that of the control.

Figure 5. Western blotting of procaspase-8, -9, -3 and PARP expressed in MCF-7 cells treated with SeGLP-2B-1.
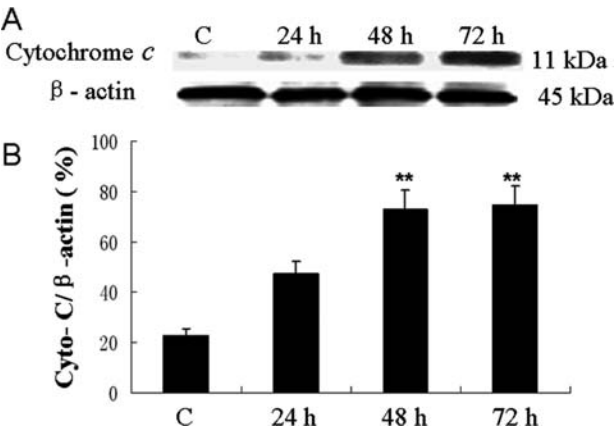

Figure 4. (A) Western blot analysis of the expression of cytochrome *c* in the cytoplasm. (B) Relative intensities of the protein bands were quantified by densitometry and were scaled to the ß-actin reference bands. Values are the means ± SD (n=3). **p≤0.01 compared with the control.

After 48 h, about 3-fold increase in the cytochrome *c* level was observed (Fig. 4). These results indicated that SeGLP-2B-1 induced apoptotic cell death via a mitochondria-mediated pathway.

Effect of caspase on SeGLP-2B-1-induced apoptosis in MCF-7 cells. Release of cytochrome *c* from mitochondria can trigger a series of events leading to the activation of effector caspases. To investigate the role of caspases in SeGLP-2B-1-induced cell apoptosis, the effects of SeGLP-2B-1 on the expression of caspase-8, caspase-9, caspase-3 and PARP in MCF-7 cells were determined by Western blotting. SeGLP-2B-1 induced the degradation of procaspase-8, procaspase-9 and procaspase-3 and PARP cleavage in a time-dependent manner (Fig. 5). PARP was cut into small fragments of 83 kDa by activated caspase-3, and with time, the expression of 83 kDa-specific fragment gradually increased. To further examine the participation of caspases in SeGLP-2B-1-induced apoptosis, we used the specific fluorogenic peptide substrates IETD-pNA, LEHD-pNA and DEVD-pNA to detect caspase-8, caspase-9 and caspase-3 activities, respectively. The results indicated that caspase activity was significantly increased in a time-dependent manner after the cells were treated with
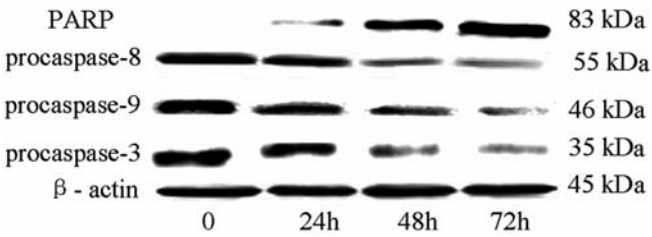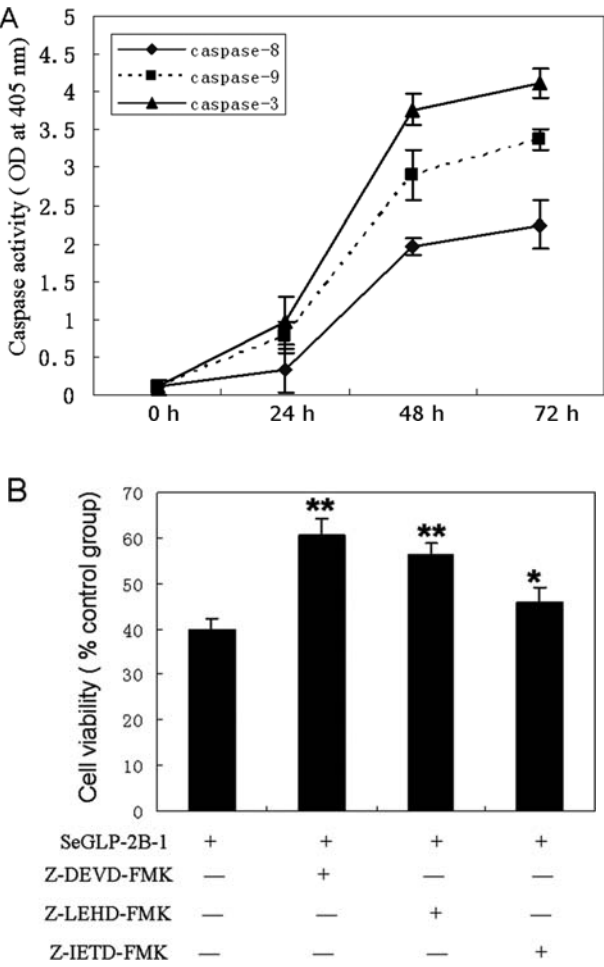

Figure 6. Effect of caspases on apoptosis induced by SeGLP-2B-1 in MCF-7 cells. (A) Activities of caspase-3, -8 and -9 of MCF-7 cells treated with SeGLP-2B-1. (B) Effect of caspase inhibitors on SeGLP-2B-1-induced apoptosis in MCF-7 cells. The cells were cultured in the absence or presence of caspase inhibitors. At 2 h prior to the addition of SeGLP-2B-1, caspase inhibitors were added and the cells were further incubated for 72 h. Data shown are the means ± SD n=3; *p≤0.05, **p≤0.01 compared with the SeGLP-2B-1 group.

0.18 μM SeGLP-2B-1 (Fig. 6A). We examined cell viability when cells were treated with SeGLP-2B-1 in the absence or presence of the caspase-3 inhibitor, z-DEVD-FMK or the caspase-9 inhibitor, z-LEHD-FMK, or the caspase-8 inhibitor, z-IETD-FMK, which were used to block intracellular proteases. Treatment with caspase-3, -8 or -9 inhibitor increased the

number of viable MCF-7 cells in the presence of SeGLP-2B-1 (Fig. 6B). Cell viabilities in the presence of no inhibitor, caspase-3 inhibitor, caspase-8 inhibitor and caspase-9 inhibitor were 39.7, 60.6, 46.0 and 56.3%, respectively. These results indicated that both caspase-3 and caspase-9 inhibitors significantly increased cell viability in the presence of SeGLP-2B-1 in MCF-7 cells ($p \le 0.01$), and also caspase-8 inhibitors increased cell viability ($p \le 0.05$).

Discussion

The goal of cancer chemoprevention is to inhibit the proliferation of cancer cells and to selectively induce cancer cell death using natural or synthetic compounds. Further understanding of the chemotherapeutic mechanisms of candidate compounds may provide a rational approach to using such agents to enhance apoptosis as a strategy for effective chemoprevention of cancer (20). Accumulating evidence supports the hypothesis that apoptosis is a critical mechanism in cancer chemoprevention by Se-containing compounds (21). The synthetic Se-enriched chemotherapeutic polysaccharide κ-selenocarrageenan might inhibit proliferation of K562/ADM cells and induce apoptosis via a Fas-caspase-3 pathway (22). A Se-protein polysaccharide obtained by water extraction of Se-enriched *Agaricus blazei* inhibited K562 cell proliferation and caused morphological changes characteristic of apoptosis, nuclear DNA fragmentation, and an increased in caspase-3 activity *in vitro*, indicating that this Se-protein polysaccharide induced apoptosis of K562 cells via a caspase-dependent apoptotic pathway (23). Our present data demonstrated that SeGLP-2B-1 decreased MCF-7 cell viability via apoptosis.

The apoptotic signal is propagated by either of two distinct mechanisms: the extrinsic pathway or the intrinsic mitochondrial apoptosis pathway (24). The extrinsic pathway involves caspase-8 activation, which then directly activates the downstream effector caspase-3, -6 and -7, leading to apoptosis. The intrinsic pathway depends on mitochondria, in which caspase-8 activation results in the proteolysis of the Bcl-2 interacting domain, causing translocation of the smaller Bcl-2 product to mitochondria where it induces the mitochondrial apoptosis pathway (25). The subsequent disruption of $\Delta \Psi m$ and release of proapoptotic molecules such as cytochrome *c* and Smac/DIABLO result in caspase-9/caspase-3 activation and apoptosis (26).

Our current study showed that caspase-8 was involved in SeGLP-2B-1-induced apoptosis as shown by an increase in caspase-8 activity and down-regulation of procaspase-8 expression. Down-regulation of procaspase-8 expression did not significantly reduce cell viability, however, suggesting that caspase-8 does not directly regulate the proteolysis of caspases during SeGLP-2B-1-induced apoptosis. On the contrary, SeGLP-2B-1 induced both time-dependent degradation of procaspase-9 and -3 and PARP cleavage, suggesting that caspase-8 activation may involve the mitochondrial pathway. Both the caspase-3 (z-DEVD-FMK) and caspase-9 (z-LEHD-FMK) inhibitors significantly blocked intracellular proteases, resulting in increased cell viability.

Disruption of $\Delta \Psi m$ and release of cytochrome *c* are critical events in both the caspase-dependent and -independent

apoptotic pathways (27). In our study, $\Delta \Psi m$ paralleled the apoptosis data and was consistent with the hypothesis that SeGLP-2B-1 induced mitochondria-mediated cell death. The results suggested that SeGLP-2B-1 caused early damage to mitochondrial membrane potential by forming pores in the mitochondrial membrane known as permeability transition pores through which a proportion of apoptotic factors were released. Release of cytochrome *c* from the mitochondria into the cytoplasm serves as a trigger to activate caspase-9. Inhibitors of caspase-3, -8 and -9 partially blocked SeGLP-2B-1-induced MCF-7 cell apoptosis. At the same time, there was an increase in caspase-3, -8 and -9 activities and PARP cleavage, indicating that caspases participated in this apoptotic process. Our results provide a potential molecular mechanism for SeGLP-2B-1-induced apoptosis of MCF-7 cells by the mitochondria-mediated pathways.

Acknowledgements

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